# Inhibition of Early Atherogenesis by Rosuvastatin in Male Rats with Diabetes Mellitus

*GUO Shu-li, CHEN Zuo-yuan△, LI Shan, Li Xia*

*(Department of Cardiology, the Affiliated Hospital of Qingdao University Medical College, Qingdao 266003, China)*

ABSTRACT Objective: To investigate the effects of rosuvastatin on early atherogenesis in male rats with type 2 Diabetes Mellitus, and to explore the mechanism of them. Methods: Forty-five male SD rats were randomly and equally divided into three groups : control group (NC group), DM group and rosuvastatin group (DR group). High fat and high glucose diet were given to establish the DM rat model. The rats in DM and DR group were fed with high-glucose and high fat diet for one month ,and were injected with streptozotocin (25mg/kg) intraperitoneally. The rats in NC group were fed with full diet, and were injected with citrate buffer. After that, DR group was given rosuvastatin 5mg/ (kg·d), while NC group and DM group were given normal saline. All the medicine was given by intragastric administration for the three groups. sixteen weeks, to measure the TC, TG, LDL-C , BG and PGI. Immunohistochemistry was used to analyze the expressions of CD40, MMP-2 and AP-1 in aortic wall. Results: The levels of TC , TG, LDL-C and BG in DM and DR groups were markedly higher than that of NC group (F=33.71～426.05，q=5.26～40.82，P<0.01), but the differences between DM group and DR group were not significant (P>0.05). The CD40, MMP-2 and AP-1 expression level in DR group and the monocytes infilitrating into the intima of the aorta was significantly lower than that of DM (F=36.86～716.82，q=8.59～37.86，P<0.05). The endothelial damage of the aorta in rosuvastatin groups was less severe than that in DM group. Conclusion: Rosuvastatin can prevent early atherogenesis by inhibiting the CD40, MMP-2, AP-1expression and alleviating the monocytes infilitrating into the arterial wall.

Key words: Diabetes Mellitus; Atherosclerosis; Rosuvastatin

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## Introduction

Diabetic angiopathy is one of chronic complications of diabetes which is the most fatal disease to diabetic patients. Atherosclerosis (AS) is a key factor to accelerate the diabetic angiopathy. Conversely, DM will accelerate the atherosclerosis process as an independent risk factor, more inportantly, DS can incur Atherosclerosis by damaging the endothelial cell. This study takes SD rats who has similar AS formation with human being as example, to observe the changes of CD40, MMP-2, AP-1 expression and endothelium infiltrating mononuclear cells number when early AS occurs. The objective is to investigate the inhibition effects of rosuvastatin on early atherogenesis in male rats with type 2 Diabetes Mellitus and to discuss the mechanism as the theoretical suport for T2DM early AS treatment.

## 1 Materials and Methods

### 1.1 Materials and apparatus

Cholesterol, sodium cholate and streptozotocin (STZ) were purchased from Qidong Tongxin Biochemical Factory. Ether anhydrous was purchased from the Affiliated Hospital of Qingdao University Medical College. EliVision kit was purchsed from Fuzhou Maixin Biological Technology Co., Ltd.. CD40, MMP- 2 and AP-1 polyclonal antibody was purchased from Wuhan Boster Biological Engineering Co., Ltd.. Rosuvastatin was provided by the AstraZeneca. Blood Glucose was determined by glucose oxidase, while plasma insulin was determined by RIA kit (CIS). Triglycerides, Cholesterol and LDL (low-density lipoprotein) cholesterol were determined by Hitachi automateic biochemical analyzer.

Forty healty male SD rats at clean grade were provided by Laboratory aniaml center of Qingdao University Medical college. The rats were 8-week-old, and the weight is 180-220g. Every four rats were raised in one standarized case. The rats took food and drunk freely. Twelve hours sunshine per day was provided.

### 1.2 Method

**1.2.1 Animal grouping and AS rat model establishing** The rat model who had early atherogenesis in atery was established by high-fat, high-sugar feeding[2]. Forty-five SD rats were divided randomly into three group. Group A was the rats with normal control (NC Group). Group B was Type 2 diabetes mellitus (DM Group). Group C was the rats with T2DM intervened with Rosuvastatin (DR Group). There was 15 rats for each group. Group B, and GroupC were feeded with high-fat and high-sugar food (10% lard, 20% sucrose, 2.5% cholesterol, 1% sodium cholate and 66.5% conventional feed) and fructose water (12%). One month later,the rats were shot sigle dose of 25mg/kgSTZ(made by 0.1mol/L citrate buffer preparation with pH4.2, the concentration is 0.25%) intraperitoneal injection after fasting for 24 hours. Group A was shot citrate buffer as control modeling. Group C was taken intragastric administration with 5mg/kg·d Rosuvastatin at morning and night

Author introduction: GUO Shu-li,(1982-), female, master, Mainly engaged in cardiology research

△Correspoonding author: CHEN Zuo-yuan,

E-mail：chenzuoyuan@sohu.com

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per day for 12 weeks. Group A and B were taken intragastric administration with saline as control. All rats were raised in separate cages in clean grade animal feeding standard. The probation last 16 weeks.

**1.2.2 The detection on blood lipid, blood glucose and insulin** Fasting blood samples were taken from left ventricular after the probation was completed, and were centrfuged for 10min at 3000rpm. After that, the concentration of steady-state blood glucose (BG), steady-srate insulin (PGI) and the level of total cholesterol (TC) triglycerides (TG), and low-density lipoprotein (LDL) cholesterol were determined on automateic biochemical analyzer.

**1.2.3 Pathomorphological examination** When the rats were induced 1% pentobarbital sodium anesthesia with intraperitoneal injection, a left chest incision. were made. After that, the left ventricular was lavaged with 0.85% sodium chloride till the fluid outflew from injured right atrial appendage became clean. Then an artery with length of 0.5cm was sheared off an artery with length of 0.5cm from thoracic aortic, and which was fixed it into 10% buffered formaldehyde solution for one night. The cross section of the paraffin embedded artery was cut into serial sections with thinckness of 4μ m. Then we can observe the Pathomorphological changes on the hematoxylin and eosin stainned sections.

**1.2.4 The detection on expression of CD40, MMP-2 and AP-1** When the experiment completed, the rats were killed with overdose sodium pentobarbital anesthesia. The lesion vessel were cut into four or five sections equally, and then was fixed into 10% buffered formaldehyde solution. The cross section of the paraffin embedded artery was cut into serial sections with thinckness of 4μ m. Then cut the sections at every 100μ m into 3 slices. The CD40, MMP-2 and AP-1 immunohistochemical staining was done by P-S method, DAB display, and hematoxylin stainning. AP-1 antibody shew positive when brown particulate matter appeared in the cell nucleus of vascular endothelial. CD40 and MMP-2 shew positive when cell membrane and cytoplasm stained yellow. The positive cells and endothelial cells were recorded in the same slice under high power microscope.The percentage of positive cell to endothelial cell shew the positive rate of CD40, MMP-2 and AP-1.

**1.3 Statistical analysis**

Statistics software SPSS11.0 was used for analysis. All data was expressed as $\bar{x}\pm s$. Variance analysis was applied in comparison between multiple groups, and Q-test was applied in comparison between two groups. We set $P<0.05$ as statistically significant difference index.

## 2 Results

**2.1 The result of the detection on blood lipid, blood glucose and insulin**

The level of TC, TG, LDL-C and BG of DM group and DR group was significantly higher than that of NC group ($F=33.71\sim426.05$, $q=5.26\sim40.82$, $P<0.01$), while the level of PGI had no significant differnce ($P>0.05$). The level of TC, TG, LDL-C and BG of DR group was lower than that of DM group, and the variance was obvious ($F=10.80\sim92.93$, $q=4.65\sim13.63$, $P<0.05$). (Table 1)

Table 1 The comparison on serum index between groups($n=15$, $\bar{x}\pm s$)

| Group | TC (c/mmol·L-1) | TG (c/mmol·L-1) | LDL (c/mmol·L-1) | BG (c/mmol·L-1) | PGI (ρ /μ g·L-1) |
|---|---|---|---|---|---|
| NC group | 0.81± 0.07 | 0.79± 0.07 | 0.74± 0. 07 | 6.18± 0.84 | 16.8± 0.74 |
| DM group | 1.36± 0.1* | 1.78± 0.11* | 0.88± 0.08* | 26. 81± 4.36* | 17.4± 0.69 |
| DR group | 1.22± 0.1*# | 1.42± 0.10*# | 0.65± 0.07*# | 20.68± 3.70*# | 16.8± 0.8 |

Compared with NC group: F=33.71~426.05, *q=5.26~40.82, P<0.01

Compared with DM group: #F=10.80～92.93，q=4.65～13.63, P<0.05

**2.2 Histomorphological changes**

Observed with naked eye, the endometrial of the rats in NC group was smooth. The endometrial of the rats in DM group had a few of yellow fat-like plaque protruding into the lumen.These focal plaque were in patchy distribution near the aortic arch. Observing under the microscope, the thorough endothelial cell can be seen in NC group. The monolayer clung to the elastic plate.The tunica elastica was in uniform thickness, and almost of them were contracted smooth muscle cells. While in DM group, the auxetic endothelial cell were partly shed with increased junction gap. There was some adhesive monocyte on the surface of endotelial. Monocyte infiltration appeared in intima. Macrophages and fat vacuoles could be seen under the microscope. The mitochondrial was swelled in cytolasmic.There were synthetic smooth muscle cells in tunica elastica. The lesion in DR group was similar with that in DM group, but was not as severe as that in DM group. The intima was almost thorough and smooth.The adhesive and infiltrating monocyte into intima was less than that in DM group. The intimal thickening was found significantly lighter than that of DM group, but there wasn't obvious differences between the two Groups.

**2.3 The changes on immunohistochemical indicators**

There was almost no brown granules or few positive expression in NC group (Figure 1). There was a lot of positive brown granular substance in DM group. Some of them fused into slices (Figure 2), and the positive rate of DM group was significantly

higher than that in NC group. The positive grown granular substance in DR group was more than that in NC group, but obviously less than that in DM group (Figure 3).

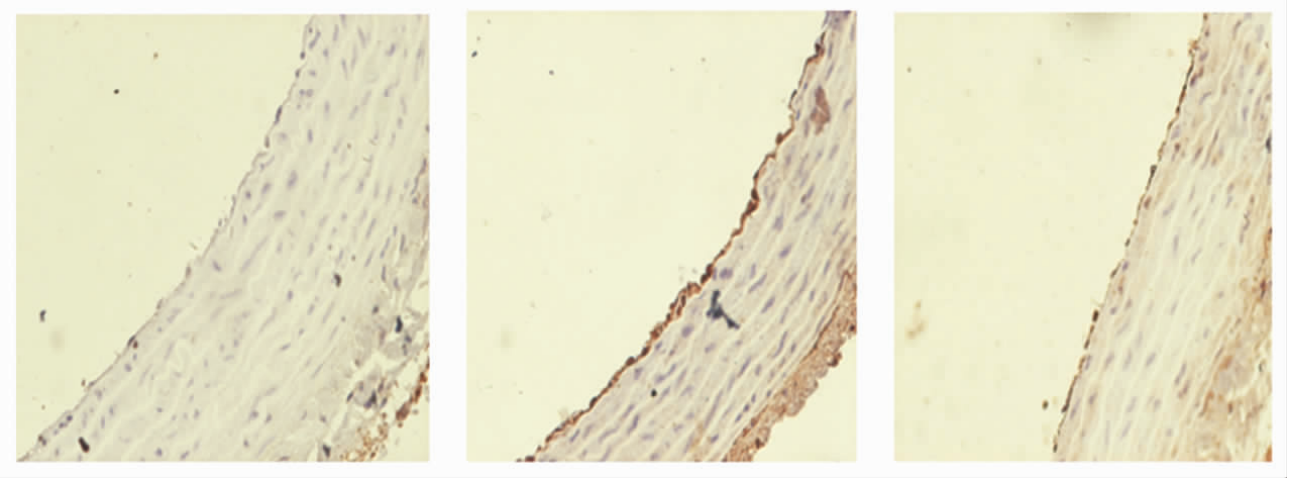

Fig1 The CD40 Expression of Aortic Wall (* 200)

The indicator of CD40, MMP-2 and AP-1 of NC group was (7.82± 0.51)%, (2.72± 0.36)%, (9.46± 5.26)%. while which was (28.84± 1.91)%, (17.14± 1.58), (48.34± 8.45). for DM group and was (12.78± 1.32), (12.18± 2.05), (28.64± 9.25) for DM group, there was significant difference between the DM group, DR group and NC group (F=91.92～961.84, q=9.48～59.35, P<0.01). The expression of DR group is obviously lower than that of DM group (F=36.86～716.82, q=8.59～37.86, P<0.05). (Table 2)

Table 2 The positive rate comparison of CD40, MMP-2 and AP-1 ( / %, $\bar{x}$± s )

| Group | qty | CD40(%) | MMP-2(%) | AP-1(%) |
|---|---|---|---|---|
| NC group | 15 | 7.82± 0.51 | 2.72± 0.36 | 9.46± 5.26 |
| DM group | 15 | 28.84± 1.91* | 17.14± 1.58* | 48.34± 8.45* |
| DR group | 15 | 12.78± 1.32*# | 12.18± 2.05*# | 28.64± 9.25*# |

Compared with NC group: F=91.92～961.84, *q=9.48～59.35, P<0.01

Compared with DM group: #F=36.86～716.82, q=8.59～37.86, P<0.05

## 3 Discussion

Atherosclerosis is the major pathological feature of diabetic vascular disease. In recent years, the prevalence of type 2 diabetes increased year by year. Atherosclerosis occurs severely earlier on diabetic patients with poor prognosis and rapid progression[3]. The probability of heart disease, cerebrovascular disease occured among Diabetes patients is 2-4 times than that of non-diabetic population[1].

CD40 is the main condition to activate lymphocytes, The interaction of CD40 and its ligand CD40L may produce a series of immune and inflammatory response, which may promote the adhesion molecules, cytokines such as matrix metalloproteinase -2 (MMP-2), and the chronic inflammatory process of AS, and ultimately leading to plaque rupture, AP-1 is an important transcription factor prevalented in cells that can enhance the transcription betweeen many kinds of matrix metalloproteinases (MMPs) and inflammatory factors. MMP-2 is the member of protease superfamily on which zinc depends, MMP-2 can degrade the matrix fibers, which can weaken the fibrous cap in plaques. It also can promote the fragility of patches, and finally lead to the rupture of plaques and thrombosis. Thus CD40, AP-1, and MMP-2 were involved in the formation of early AS. Recent studies show that, AS plaque formation process was closely related to immune and inflammatory responses, which performs as deterioration, leakage and proliferation. Various risk factors may damage the vascular endothelial cell at the start-up period, since DM was associated with hyperglycemia, hyperlipidemia, hyperinsulinemia which may increase the oxidative capacit, and damage the vascular endothelial cells. Therefore, the low density lipoprotein in plasma may permeate through the injured endothelial cells, and then accumulate in subcutaneous space. The oxidized low density lipoprotein caused the activation of endothelial cell. The results are inducing inflammatory response, promoting the proliferation and migration of smooth muscle cell, promoting the oxidation and transference of low density lipoprotein cholesterol, affecting mechanisms such as fibrinolysis, and being involved in the occurrence and development of AS. Above results showed that, the activation of CD40 and AP-1 may increase the MMP-2 expression of endothelial cells, which may be one of the mechanisms incurring diabetes

atherosclerosis and plaque rupture. Therefore, in the treatment, the transcription of matrix metalloproteinases (MMPs) and inflammatory factors were reduced by inhibiting the activity of CD40, AP-1, which can delay the occurrence and development of atherosclerosis, and keep the stability of plaque.

In this study, SD diabetic rats model were established by high lipid and high glucose diet. The morphological observation shew that the DM rat had significant damage on aortic endothelial, a large number of adhesive and infiltrative monocyte were found in the intima, and vascular smooth muscle cells (VSMCs) were proliferated. These are the characteristics of early AS. The detection of blood glucose and lipid levels showed significantly the increase of blood glucose associated with the reduction of insulin sensitivity. It indicated a successful modeling of type 2 diabetes. The serum TC, TG, LDL-C levels of DM group and DR group were higher than that of NC group obviously ($P < 0.01$). The serum TC, TG, LDL-C and blood glucose levels of DR group were singnificantly lower than that of DM group. That means rosuvastatin can regulate glucose and lipid metabolism. Aortic Immunohistochemistry showed that: compared with NC group, the vessel wall CD40, AP-1 expression of DR group and DM group increased significantly. It indicates that a substance serves as the activator to increases the expression of CD40 and AP-1 in the formation of AS. It has been reported that, high cholesterol making the expression of CD40 and AP-1 increasing was an activator of CD40 and AP-1 in the formation of AS. In this experiment, the plasma TC, LDL levels, CD40 and AP-1 expression of DM group was significantly higher than that of NC group. This result also confirmed this point. In addition, the MMP-2 expression and infiltrated monocyte of DR group was significantly less than that of DM group, but still higher than that in Group NC. It proved that rosuvastatin can inhibit the VCAM-1 and ICAM-1 expression of endothelial cell, infiltration of monocyte macrophages, phenotype transformation and proliferation of VSMCs, and reduce the vascular inflammation by inhibiting CD40 and AP-1 and indirect inhibition of MMP-2 expression. Thereby rosuvastatin can prevent the endothelial cells from lipid peroxidation to resist early AS efficiently.

In this experiment, high-lipid and high-glucose diet were took as the intervention factor. Therefore, hyperglycemia and hyperlipidemia can be an activation factor of CD40 and AP-1. The blood glucose and lipid levels, CD40 and AP-1 expression of DR groups was singnificantly lower than that of DM group. This result means that rosuvastatin can inhibite the expression of VCAM-1 and ICAM-1 in vascular endothelial cells by inhibiting CD40 and AP-1 and indirect inhibition of MMP-2 expression, which may be one of the mechanisms of its resistance to AS. In addition, rosuvastatin has various useful biological effects such as lowering blood fat, improving insulin resistance and reducing inflammation. In conclusion, the aortic CD40, MMP-2, AP-1 expression of DM rats has relationship with inflammation. Rosuvastatin has the function of lowering blood lipid cholesterol, reducing the formation of foam cell, and reducing the release of inflammatory factor. Thus it can inhibite the inflammatory response and maintain the stability of plaque. Lipid lowering medicine such as rosuvastatin is not only a lipid lowering drug, which is also expected to be the anti-inflammatory medicine. We will continue to do further studies to explore the other mechanisms of rosuvastatin's anti-AS function.

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# 瑞舒伐他汀对糖尿病大鼠早期动脉粥样硬化形成的影响

郭淑丽 陈作元△ 李 姗 李 霞

（青岛大学医学院附属医院心内科 山东 青岛 266003）

摘要 目的：观察他汀类调脂药物瑞舒伐他汀（Rosuvastatin）对 2 型糖尿病（type 2 diabetes mellitus,T2DM）大鼠早期动脉粥样硬化形成的影响，并探讨其可能的机制。方法：将 45 只雄性 SD 大鼠随机分为正常对照组(NC 组)、2 型糖尿病组(DM 组)、2 型糖尿病瑞舒伐他汀治疗组(DR 组), 每组 15 只。以喂高糖高脂饮食方法建立 SD 大鼠糖尿病模型, DM 组、DR 组给予高糖高脂饮食 1 个月后腹腔注射 25mg/kg 链脲佐菌素;NC 组给予普通饮食, 注射枸橼酸缓冲液作为对照。在此基础上,DR 组给予瑞舒伐他汀 5mg/(kg·d)灌胃,NC 组、DM 组给予生理盐水灌胃。16 周后测定各组大鼠总胆固醇(TC)、三酰甘油(TG)、低密度脂蛋白胆固醇(LDL-C)水平与稳态血糖(BG)、稳态胰岛素(PGI)浓度,用免疫组化法检测主动脉血管壁白细胞分化抗原 40（cluster of differentiation 40, CD40）及基质金属蛋白酶 -2（MMP-2）、激活蛋白 -1(activator protein-1,AP-1)的表达水平。结果：DM 组、DR 组 TC、TG、LDL-C 与 BG 水平较 NC 组均显著升高(F=33.71~426.05，q=5.26~40.82，P<0.01), 但 2 组间各指标比较差异无显著性(P>0.05)。DR 组 CD40、MMP-2、AP-1 表达水平和浸润的单核细胞数明显低于 DM 组(F=36.86~716.82，q=8.59~37.86,P<0.05), DR 组主动脉内皮损伤明显轻于 DM 组。结论：瑞舒伐他汀能抑制 CD40、MMP-2、AP-1 表达和单核细胞浸润,防止早期 AS 形成。

关键词：2 型糖尿病；动脉粥样硬化；瑞舒伐他汀

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作者简介：郭淑丽，（1982-），女，硕士，主要从事冠心病方面的研究

△通讯作者：陈作元，（1962-）男，硕士研究生导师，

E-mail: chenzuoyuan@sohu.com

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